GEORGIA INSTITUTE OF TECHNOLOGY
Engineering Experiment Station

PROJECT TERMINATION

Date 12/3/69

PROJECT TITLE: A Study of Molecular Coating of Intravascular Surfaces

PROJECT NO: B-254

PROJECT DIRECTOR: D. S. Harmer

SPONSOR: Piedmont Hospital - through a grant from the John A. Hartford Foundation, Inc.

TERMINATION EFFECTIVE: October 31, 1969

CHARGES SHOULD CLEAR ACCOUNTING BY: November 30, 1969

Overrun to be charged to Division accounts.

Nuclear & Biological Sciences Division

COPIES TO:

Project Director
Director
Associate Director
Assistant Directors
Division Chief
Branch Head
Accounting
Engineering Design Services
General Office Services
Photographic Laboratory
Purchasing
Report Section
Library ✓
Security
Rich Electronic Computer Center

ES 402 (R10-62)

B-254

M60 2

ANNUAL PROGRESS REPORT TO JOHN A. HARTFORD FOUNDATION

PROJECT B-254

A STUDY OF MOLECULAR COATING OF INTRAVASCULAR SURFACES

W. L. BLOOM, D. S. HARMER, M. F. BRYANT, and S. S. BREWER

1 July 1963 to 30 June 1964
Issued 1 July 1964

Joint Investigation from
The Ferst Research Center,
Piedmont Hospital and the
Georgia Institute of
Technology
Atlanta, Georgia

1964

Engineering Experiment Station
GEORGIA INSTITUTE OF TECHNOLOGY
Atlanta, Georgia

REVIEW
PATENT 7-10 1964 BY
FORMAT 7-10 1964 BY

GEORGIA INSTITUTE OF TECHNOLOGY
Engineering Experiment Station
Atlanta, Georgia

ANNUAL PROGRESS REPORT

PROJECT B-254

A STUDY OF MOLECULAR COATING OF INTRAVASCULAR SURFACES

By

W. L. BLOOM, D. S. HARMER,
M. F. BRYANT, and S. S. BREWER

1 JULY 1963 to 30 JUNE 1964
Issued 1 July 1964

Performed for
JOHN A. HARTFORD FOUNDATION
New York 17, New York
Joint Investigation from
THE FERST RESEARCH CENTER,
PIEDMONT HOSPITAL and the
GEORGIA INSTITUTE OF TECHNOLOGY

TABLE OF CONTENTS

This report contains 25 pages.

LIST OF FIGURES

I. INTRODUCTION

It has been the object of this study to amplify and extend our studies on molecular coating of blood vessel surfaces and blood formed elements. The initial work on blood vessel coating in the prevention of vascular thrombosis has been published[1] and our efforts have been rapidly expanded as a result of the John Hartford Foundation grant. The purpose of the study is to gather more experimental evidence on the theory of *in vivo* and *in vitro* coating of formed elements and vascular surfaces. At the same time, we have been developing methods which could modify the dextran molecule and thus give us a better insight into the nature of the mechanism by which the molecule binds to the surface of the cell. Physical measurements have been undertaken to check the presence of the molecules on the cell surface. Methods employed thus far have been electron microscopy and use of the Coulter Counter. During the major part of this year, we have been involved in building sufficiently sensitive equipment and assembling this equipment so that we may continue our use of the existing radioactive dextran. In the meantime, we have worked out methods whereby we can tag with radioactive material other dextran molecules and possibly increase the amount of radioactivity in the dextran molecule. Work has continued on the use of dextran in the prevention of intravascular thrombosis and investigators in other parts of the country have confirmed our original observations[2]. Our studies have been extended to include the study of thrombosis in the venous as well as the arterial systems and a group of patients with thrombophlebitis have been studied and will be reported at a later date.

II. CURRENT STATUS OF THE RESEARCH

A. Equipment design and development

In order to detect the levels of radioactivity expected from Carbon-14 labeled dextran, it was necessary to develop a low level counting facility. These levels will be in the range from a few tens of counts per minute (CPM) down to a few hundredths CPM. Since conventional radiation detectors capable of quantative detection of the low energy radiations from Carbon-14 have background counting rates of the order of 10 - 1000 CPM, it is necessary to provide an additional means of reducing the background so that statistically significant determinations of the Carbon-14 levels may be made.

A large part of the low-energy background radiation can be removed with adequate shielding which itself contains little or no inherent radioactivity, but the limiting backgrounds come from high energy cosmic rays which can only be removed by suitable anticoincidence shielding (i.e. surrounding the detector by other detectors which cancel out the count in the central detector if the external detectors indicate the radiation came from outside the shield).

In the facility under construction, a shielded volume six inches in diameter by forty-eight inches long has been provided so that several central Carbon-14 counters may be operated simultaneously, and also so that counters of various sizes may be used. This central volume is surrounded by thirteen N. Wood Cosmic-ray Proportional Counters used in parallel as the anticoincidence shield. These counters are 2" diameter x 52" long and contain P-10 (90% Ar - 10% CH_4) gas at 45 cm pressure. The active length is 48 inches. Each of the counters is provided with a special low-noise preamplifier and high-voltage decoupler and adjustment system so that the plateau of each counter can be

measured independently of the other counters. To minimize noise pickup and input capacity, these circuits are placed as close as possible to the counters (i.e. inside the iron shield). The outputs from the preamplifier are coupled together and a single cable carries the anticoincidence signal to the ring amplifier, a Hamner N-340 R-C - coupled linear amplifier.

Surrounding the ring counters is an electrostatic and radiation shield consisting of one inch of iron and four inches of lead.

Signals from the central Carbon-14 detectors are amplified and fed into a special scaler which contains an anticoincidence circuit which prevents registering a count if one or more of the ring counters has also fired at the same time; both the "raw" and "connected" count from the central counter are recorded by the scaler. This is done so that the performance of each counter may be constantly monitored. A block diagram of the system is shown in Figure 1. The anticoincidence shield and preamplifier system is shown in Figure 2.

The electronic circuits are essentially complete and are being tested currently. Fabrication of the iron shield has been completed and the lead-iron shield installed. Testing and calibration of the equipment should be completed by September 1. A severe delay in this part of the program occurred because of the delay in acceptance of the Frank H. Neely Nuclear Research Center where the low-level facility was to be constructed and installed. The building was to be ready for occupancy in March 1963, but actually, because of construction delays, was not ready until March 1964. However, the intervening period was spent both in design studies and in pursuing other techniques for determining the mechanism of the interaction of dextran in vivo and in vitro. These studies are described below.

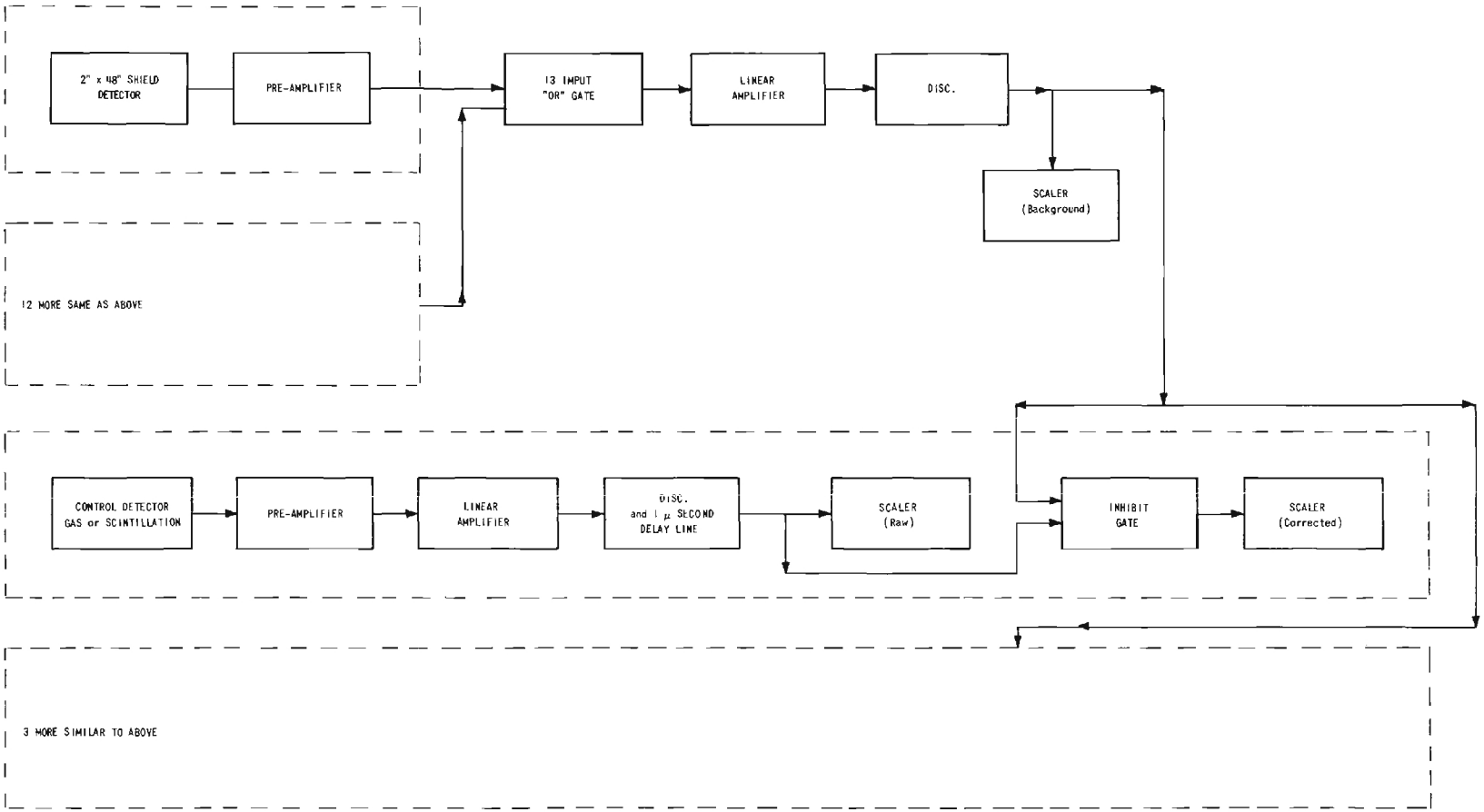
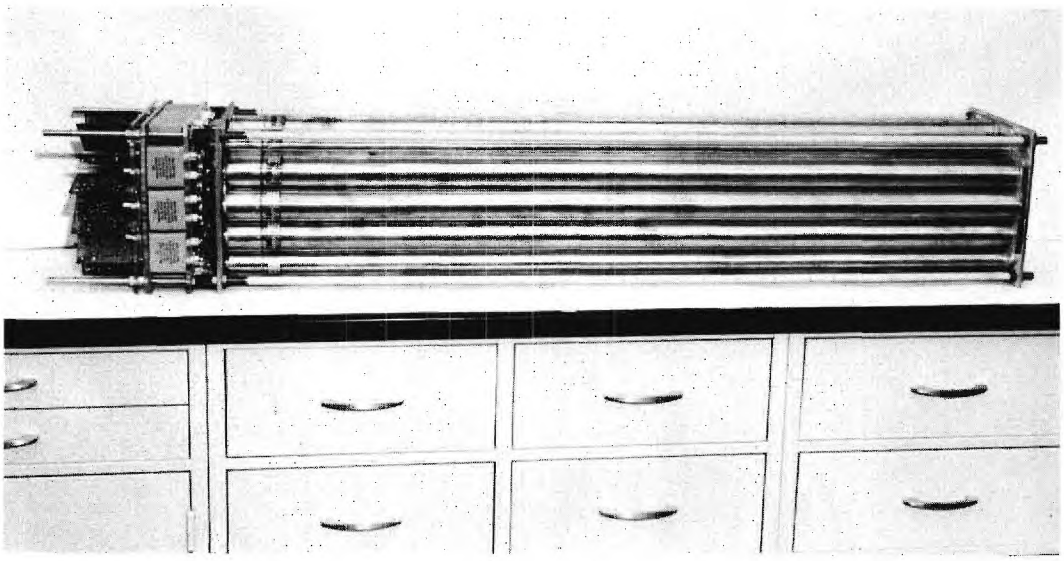
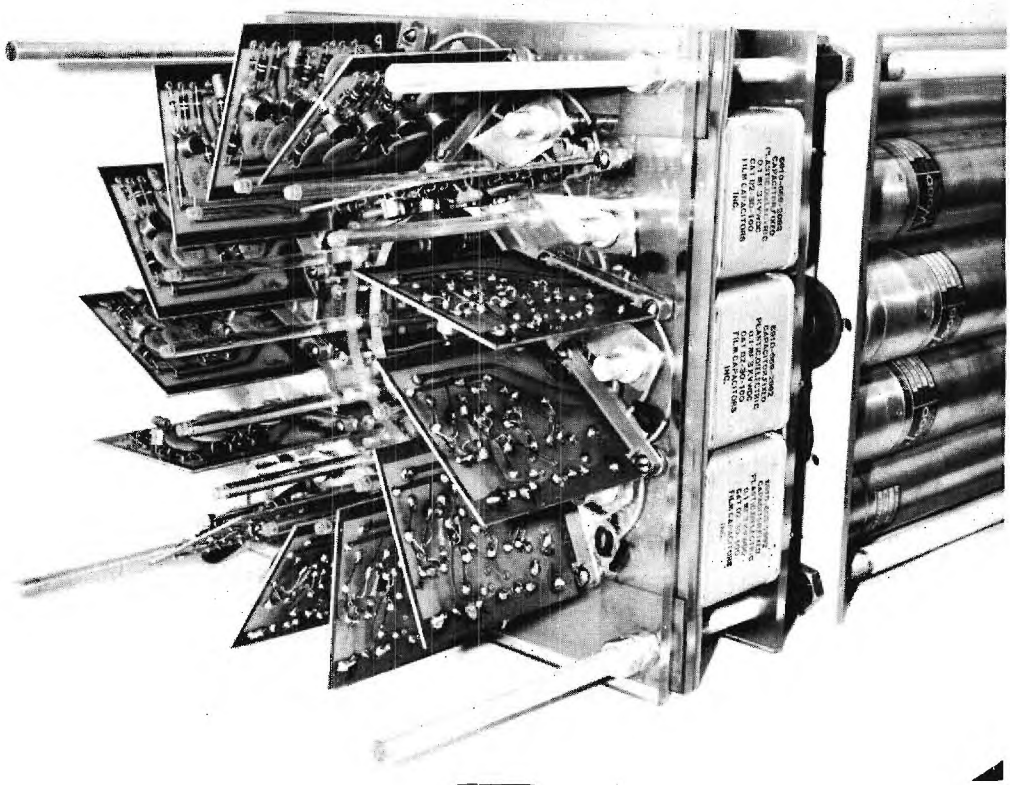


Figure 1. Block diagram of the low level counter system.



FRONT VIEW



SIDE VIEW

Figure 2. Anticoincidence shield and preamplifier system for low level counter system.

B. Coulter counter studies

If dextran is absorbed on the surfaces of the formed elements in the blood, it is possible that it might change the apparent size of the cells. This size change should be particularly apparent in the platelets where the effective size might double if the dextran molecules are absorbed "end on" in monomolecular form, as would be expected from our preliminary studies. It is possible also that no size change would occur since the dextran could conceivably replace other molecules already surface absorbed; but, in an attempt to study the antithrombotic mechanism, it is of interest to examine this possibility.

The Coulter counter is a device that measures particle volume by measuring the change in current that occurs as a particle, suspended in a conducting solution, passes through a small orifice with a current applied across the orifice. The amplitude of the current decreases in proportion to the volume of solution displaced by the particle. In the normal use of the Coulter counter, as in sizing of red blood cells clinically, the number of particles having amplitudes greater than a preset threshold are counted by adjusting the threshold to various standard incremental values. An intergral size distribution can be determined; the differential size distribution can be obtained from this by subtraction of successive counts with increasing threshold. This method is somewhat time consuming and has limited statistical accuracy and resolution (i.e. usually twenty intervals are chosen giving twenty points on a distribution curve, red blood cells may cover only 5 to 10 of these points).

To increase the accuracy and resolution of the measurements and reduce the time required to make a measurement, an instrument, widely used in experimental nuclear physics could be used in connection with the Coulter counter

detector. This instrument, the pulse height analyzer, determines the amplitude distribution of electronic pulses giving a record of the number of pulses having amplitude E within dE. The particular instrument available in our laboratory has 400 channels, and a maximum count of 10^6 in each channel permitting a resolution of 1 part in 400 and a statistical accuracy of 0.1% maximum.

It was of interest to apply this instrument to the dextran absorption problem, particularly in view of the small changes in size that might occur with red blood cells on absorption of dextran. However, the pulse from the Coulter counter differs in shape and duration from that which the pulse height analyzer is designed to measure. An adapter circuit, designed to convert the pulse into a suitable form has been built.

The Coulter Counter Adapter Circuit (Figure 3) is an electronic means of interconnecting a Coulter counter and a Technical Measurements Corporation (TMC) Model 404 Pulse Height Analyzer (PHA) for cell size distribution analysis. The Coulter counter converts the passage of a cell through its aperture to a pulse whose amplitude is proportional to the cell volume. The rise time and width of this pulse is not compatible with the requirements of the TMC PHA and the adapter is required for proper pulse shaping.

The TMC PHA requires an input pulse having a minimum amplitude of approximately 50 millivolts with a rise time not greater than 1 microsecond, a decay time of approximately 3 microseconds. The Coulter counter does not have available an output of such specifications and, therefore, some method of obtaining and shaping the pulse output from the Coulter counter is needed. We elected to sample the pulse from the Coulter counter at the plate of tube V-111, pin 6. This point gives a pulse having a rise time of 12 microseconds and an amplitude of -5 to -20 volts. The pulse is capacity coupled into the base of Q7, a 2N188

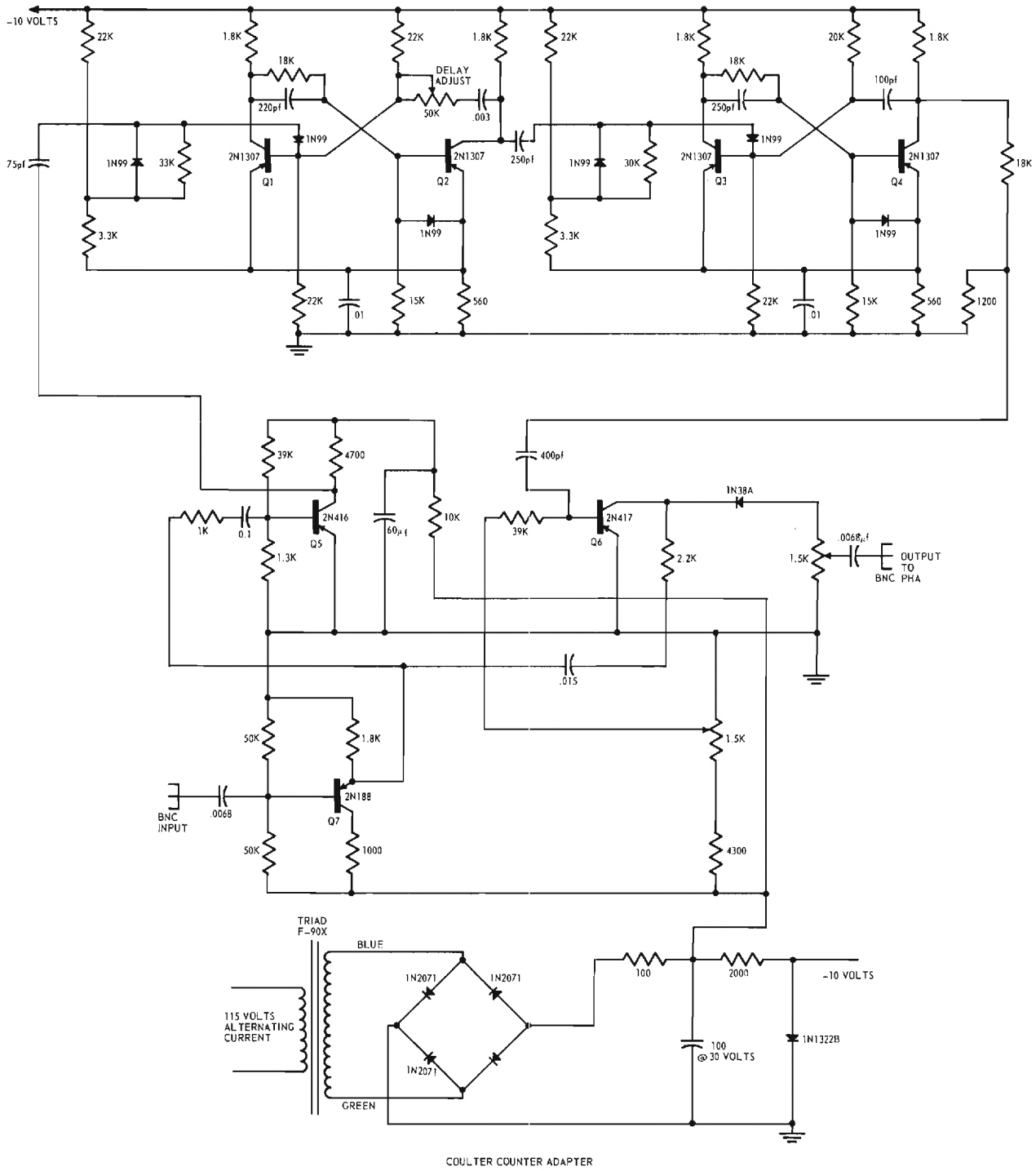


Figure 3. Coulter counter adapter circuit.

transistor employed as an emitter follower and having a gain of 0.8. The emitter follower provides impedance matching between the Coulter counter and the relatively low input impedance of Q6, a 2N417 transistor which functions as the gate. The output of the emitter follower is split and fed to (1) the gate input and (2) an amplifier, Q5, of the grounded emitter type having a gain of -30. That portion of the pulse that drives the amplifier is of sufficient amplitude to drive the amplifier to saturation during the first 0.1 microsecond of its rise. The common emitter configuration of Q5 produces a phase inversion of 180° and with saturation occurring within 0.1 microsecond of the start of the pulse, the output of this stage is a positive square wave of 8 volts amplitude and 0.1 microsecond rise time. The square wave thus obtained is differentiated by the 75 picafarad coupling capacitor between Q5 and Q1 yielding a positive and negative pulse of short duration. The 1N99 diodes in the base circuit of Q1 permit only the positive pulse to reach Q1.

The 2N1307 transistors, Q1 and Q2, form a one shot multivibrator that functions as the delay circuit for the gate pulse. The pulse width of this circuit is made adjustable and is controlled by the Delay Adjust, a 50 kilohm potentiometer which can be varied between 4 and 15 microseconds. The output, taken from the collection of Q2, is a negative square wave, 8 volts in amplitude and 4 to 15 microseconds in duration. A 250 picafarad capacitor between the collector of Q2 and base of Q3 differentiates the square wave yielding a negative and positive pulse. The positive pulse is produced by the trailing edge of the square wave and, therefore, occurs 4 to 15 microseconds after Q1 was triggered (depending upon the Delay Adjust setting). The required delay has now been obtained and the positive pulse is used to drive the one shot multivibrator composed of Q3 and Q4. The duty cycle of this multivibrator is

fixed and result is a negative square wave at the collector of Q4 having an amplitude of 8 volts and width of 3.5 microseconds. The 3.5 microsecond square wave thus obtained is the gate pulse and drives the 2N417 gate (Q6). The gate transistor, Q6, is normally conducting and short circuits the output of Q7 through a 2200 ohm resistor thereby preventing any output to the PHA. The negative gate pulse cuts off Q6 during the sampling period and a portion of the Coulter counter signal is passed to the output BNC connector. The sample of the original signal thus obtained will be 3.5 microseconds in width but will retain the relative amplitude information. A 1500 ohm potentiometer in the output of Q6 permits amplitude adjustments for driving the PHA.

The pulse produced by the Coulter counter are essentially uniform in rise time regardless of amplitude. The Delay Adjust is set equal to this time and the sampling occurs at the peak of the input pulse.

Cell size distributions obtained in the conventional manner, and with the Pulse Height Analyzer, are shown in Figure 4. From examination of these figures the increased resolution is apparent. Using this technique, a series of studies are under way of human red-blood-cell size distributions with and without dextran. Since surface absorption phenomena are rate dependent, therefore functions of both time and concentration, a number of measurements must be made under various conditions. After completion of the red-cell studies and the development of the technique, the more difficult measurements on platelets will be instituted.

C. Electron microscopy

In a further attempt to elucidate the nature of the intravascular surface phenomena, a series of studies of vascular surfaces was begun using the electron microscope. A survey of the literature revealed no work on vascular

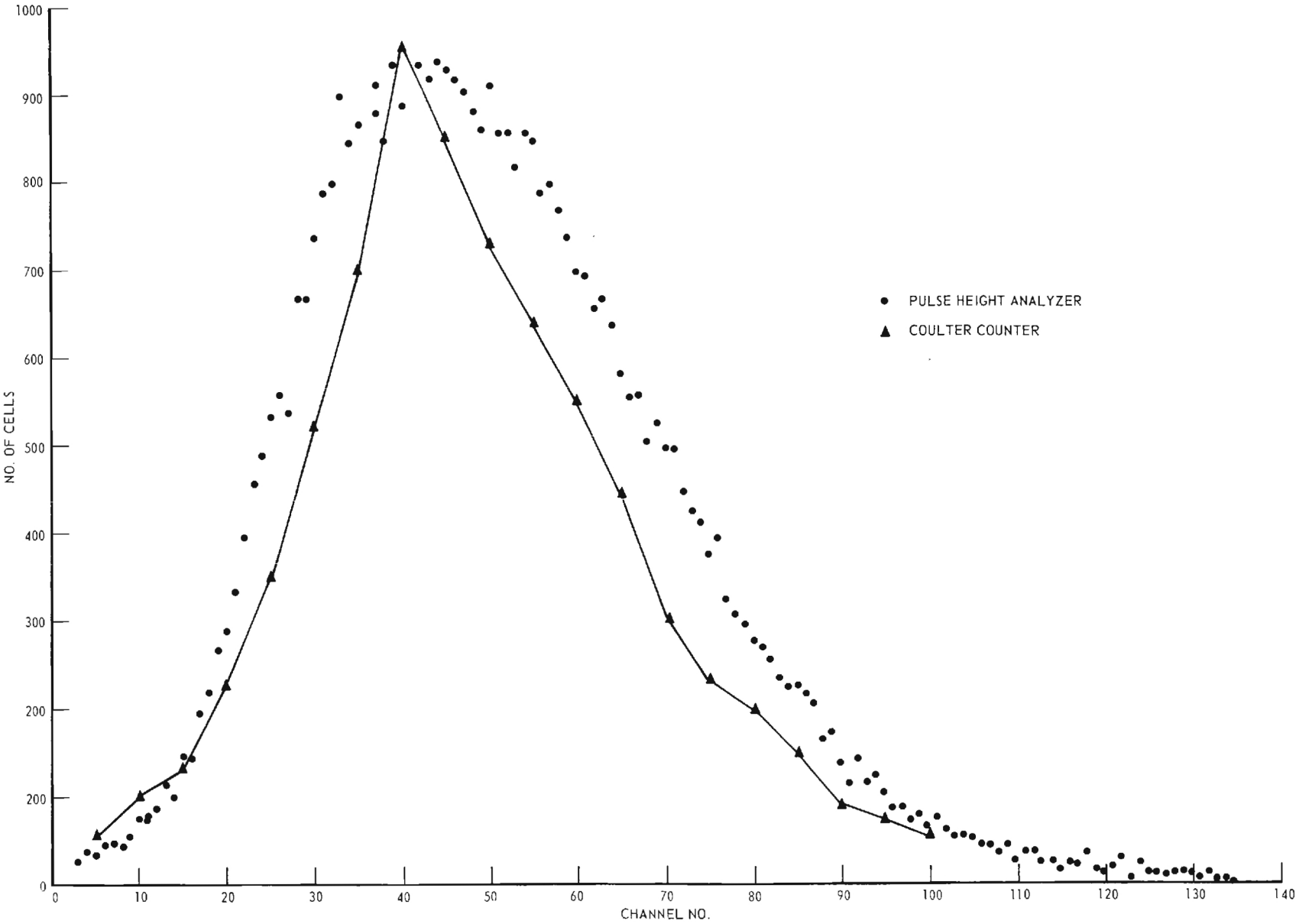


Figure 4. Red blood cell size distribution.

surfaces or on preparation methods for such tissues for examination under the electron microscope. The electron microscopists at Georgia Tech, Mr. John Brown and Mr. Jim Hubbard, felt that in view of the size of the dextran molecule it might be possible to see these molecules on surfaces at the high magnifactions and with the new techniques available with the electron microscope. Since no previous studies were available, it was of interest to see what vascular surfaces looked like at magnifications up to 115,000 times. Since dextran is water soluble, considerable care must be taken in sample preparation for the studies and some new methods of preparation need to be developed. For the preliminary studies conventional replication was used. A staining technique has also been tried.

In an attempt to see dextran on the surface of a blood vessel, two samples were taken of the femoral artery from a dog. The first segment was taken before dextran was given and the second, taken from the corresponding place on the other side of the body, was taken one hour after the admission of dextran. Part of both samples was split, opened out, and microtomed to give a thin section containing the inner layer (intina) of the artery. These microtomed sections were frozen, dried in a vacuum to preserve their form, and replicated by the Pt shadow-carbon evaporation technique.

The unmicrotomed pieces were stained with phosphotungstic acid and replicated in wet form by a two-stage plastic-carbon replication technique. No differential absorption of the stain was noted upon observation of these replicas in the electron microscope.

Attempts were made to see molecular dextran by replication of slides coated with solutions of dextran in water and dextran in methanol and allowed to dry. The results of observations of these replicas were inconclusive.

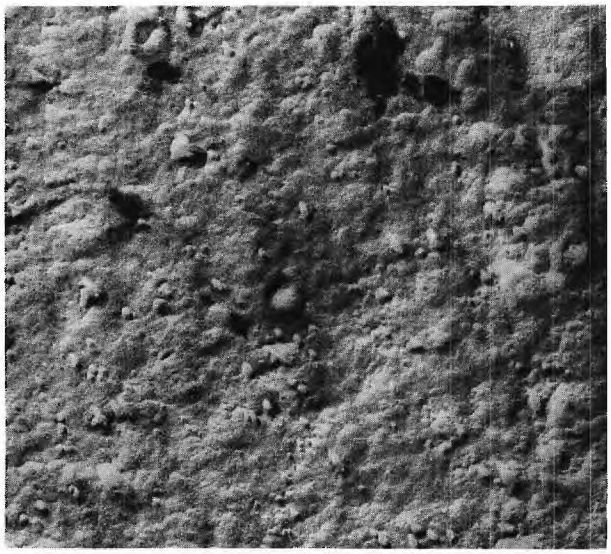
Future attempts to see dextran on the surface of a blood vessel will be made by cutting ultra-thin sections of a vessel from an "end on" orientation, and observing these sections directly in the electron microscope.

Some of the resulting electron microscope photographs of vascular surfaces are shown in Figures 5 and 6, both with and without dextran. In these figures the magnification is shown as well as a scale in microns (μ). For comparison, a red blood cell is about 8 microns in diameter and would be several times larger than the 115,000 times magnification photographs. Not reproduced here are some interesting stereoscopic photographs at high magnification.

D. Carboxy dextran preparation

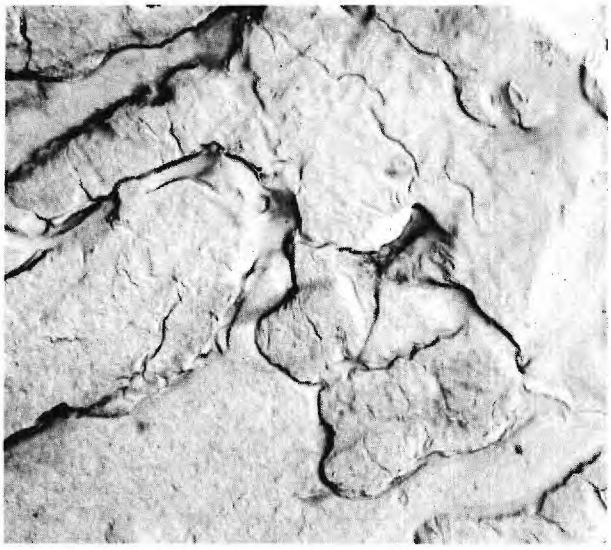
In order to improve the tracer method and to have more Carbon-14 labeled dextran available for these studies, additional preparations of labeled material were of interest, particularly if the labeled compound could be prepared with higher specific activity. In addition, it was also of interest in the mechanism studies to alter the aldehyde groups of the dextran to see if this produces a change in the anti-thrombotic effect or in the duration of *in vivo* and *in vitro* effects. It has been proposed that the aldehyde group, being very reactive and on the end of the molecule, might be the active agent in the surface bonding, particularly in view of the preliminary tracer studies[1] which indicate the molecules are surface bound "end on". Alteration of the end group to a carboxyl or an alcohol-group may change the behavior of the dextran in such a way as to provide a clue to the nature of the surface bonding mechanism.

To these ends, a compound, with addition of the carboxyl group, has been prepared. This material has been prepared by the method of Isbell[3]. This method is the classical addition of the cyanide group to the aldehyde and

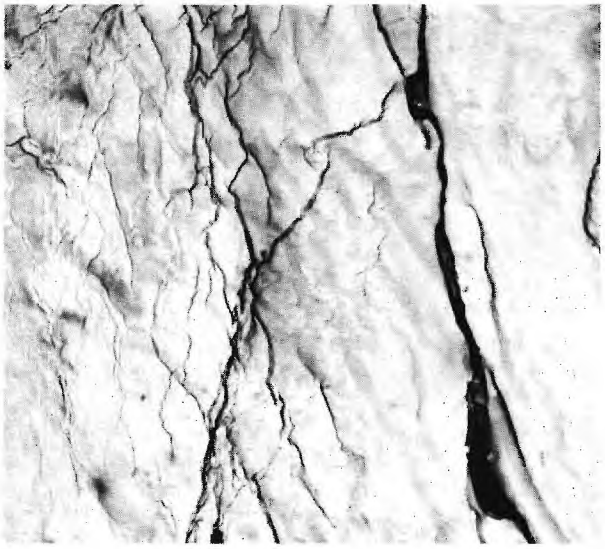


a. Platinum shadowed-carbon replica of femoral artery one hour after the admission of dextran. (48,000x)

b. Platinum shadowed-carbon replica of femoral artery without dextran. (48,000x)



c. Platinum shadowed-carbon replica of femoral artery one hour after the admission of dextran. (4,850x)

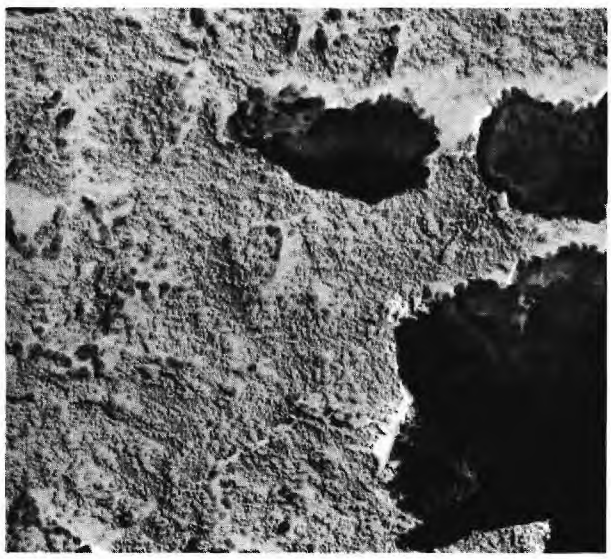


d. Platinum shadowed-carbon replica of femoral artery without dextran. (4,850x)

Figure 5.

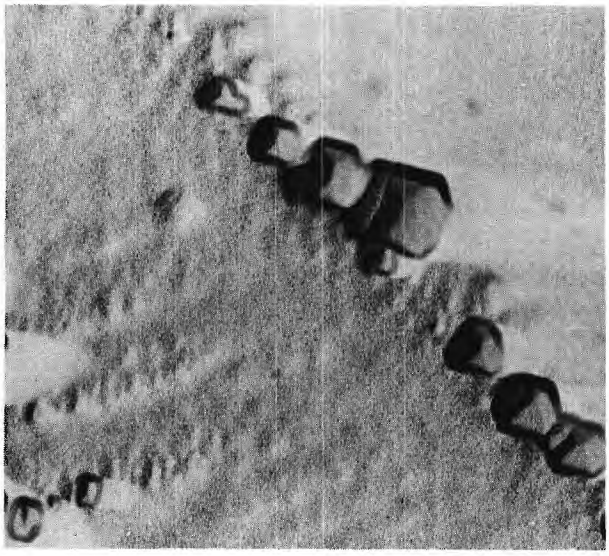
0.1μ

a. Femoral artery one hour after admission of dextran. Stained with 1% phosphotungstic acid in ethanol. (29,000x)



0.1μ

b. Femoral artery without dextran. Stained with 1% phosphotungstic acid in ethanol. (29,000x)



0.1μ

c. Platinum shawoded-carbon replica of dextran dispersed in ethanol. (48,000x)

Figure 6.

subsequent hydrolysis to the carboxylic acid. Carbon-14 labeled cyanide (as sodium cyanide) is available with high specific activity which would permit preparation of carboxy dextran with specific activities higher than that prepared by W. L. Bloom from natural sources grown in a Carbon-14 atmosphere.

Preparations of unlabeled carboxy dextran have been made to determine how the material behaves *in vivo* and *in vitro* and depending on the results of these studies, Carbon-14 labeled carboxy dextran will be prepared.

Investigation of the anti-thrombotic effect *in vitro* with the thromboelastograph with the first batch of carboxy dextran indicates that this material has a similar effect to dextran. Enough material for *in vitro* studies is currently being prepared. Since this material may change either the short term behavior or the long term persistance of the anti-thrombotic effect, a number of *in vivo* studies will be carried out in the forthcoming grant period.

E. Electrolysis studies

The formation of thrombi by the use of electric current has been studied as another means of a control situation to produce intravascular thrombosis and study the effects of dextran on this thrombosis. A review of the problem led us to study the mechanism of electrical thrombosis. In the past it had been thought that this thrombosis was caused by a change in polarity of the transmural potentials. Our findings revealed that the electrical production of *in vitro* blood clot or *in vivo* thrombus was a critically voltage-dependent phenomenon. In analogy with the electroplating processes there exist a voltage below which the coagulation did not occur. Heparinized blood or plasma does not coagulate below a potential of 2 volts. When adequate precautions are taken to assure the passage of the same quantity of electricity as that which

produced clotting at a higher voltage, thrombosis was not observed. Thus the thrombosis appears to be a function of the voltage rather than the amount of current passing through the solution of blood vessel wall. Above the threshold the amount of clot is a function of the number of coulombs passing through the medium. It was found that the potential for thrombosis was about .3 volt lower than the decomposition potential of isotonic saline. It is important that this potential is much higher than the injury potential and changes in transmural potentials which have been found to occur in vivo when the vessel wall is damaged by other means. It has thus been concluded that the initiation of normal thrombotic processes cannot be pictured as modeled by the electrical system which we have studied.

The mechanism of electrical thrombosis has been used to produce a method with a more controllable means of producing intravascular thrombi which, although not identical to the physiologic state, nevertheless provides us with a useful method for studying the effects of dextran or other molecules on the prevention of electrical thrombosis. This thrombotic model has been studied using dextran and it has been found that the dextran definitely diminishes the clot. This work has to be extended in numbers before we will be ready to report on the information. As a result of this study, a contribution was voluntarily provided by the Glancy Foundation to study the use of this method to produce experimental myocardial infarction. From this investigation, a new method of experimental myocardial infarction has been produced and this data has been accepted for publication[4]. It is our intention now to use this method in the study with our dextran project. It is important to know whether the coagulation at the positive electrode is the result of pH changes of sulfhydryl changes in the protein molecule. This is being studied by collecting and

analyzing the gas that is evolved at the electrode, by studying the effects of non-sulfhydryl containing molecules such as gelatin, and by investigating the precipitation phenomenon by interposing a cellophane membrane between the electrode and the plasma or blood.

F. The hematologic (coagulation) aspects of the investigational work centering around dextran

Previous studies had allowed us to document with Thrombelastography that the presence of dextran (especially of an average M.W. of 75,000) in the plasma produced a coagulation defect which is predictable on the basis of the quantity infused intravenously and the time elapsed following the infusion, up to eight days. During the past twelve months, we have continued the basic studies on normal human beings, patients with thrombo-embolic disease, and the mongrel dogs but have pointed the protocol toward more definitive methodology to better establish some of the hypotheses which are indicated to be fact by all reason but which still defy totally adequate demonstration. The problems center around the fact that the platelets cannot be handled for adequate study without inflicting trauma which obscures the platelet characteristics which we intend to display are being modified by the physical presence of dextran. Doctor Ponder's electrophoretic studies and measurements of zeta potentials in 1957 suggested that dextran "coated" platelets. Doctor Adelson et al in 1959 used radioisotopic labeling techniques to further suggest that platelets were "coated" by dextran. No work thus far has satisfactorily proved this point nor has anyone been able to suggest that such a phenomenon could account for any significant alteration of platelet physiology such as would explain the hemorrhagic problems hitherto encountered following the infusion of large quantities of dextran into

patients. The use of ultra-sonic energy for "sub-lethal" platelet treatment before and after dextran "exposure" came to mind as a possible useful method for displaying the alterations in the physical properties of platelets which are "coated" with dextran. The main problem here was the fact that no work has been done with ultra-sound and platelets other than total disruption and subsequent study of purely chemical properties of platelets. Our work thus far accomplished, provides us with some most provocative data to support the notion that "coated" platelets are "tougher", perhaps by reason of their encasement, since the same quantity of ultra-sound disrupts less platelets and creates a different sort of morphologic change as observed with the phase contrast microscope. Photographic techniques have had to be developed to document the differences observed. In order to supply a more carefully controlled quantity of ultra-sound, we have felt it necessary to go from the external "tank" application to the internal "micro-tip" application and much more background work must be continued before we begin to fully interpret the observations already made. There seems little doubt that this sort of approach will yield us the data which can make the heaviest inferences that platelets are indeed coated and that, thereby, they are ever so subtly altered to an extent that only their property of viscous metamorphosis is compromised. This property can be measured only with such a method as Thrombelastography wherein platelets are gently handled and, following re-calcification, allowed to proceed in their chemical and physical courses of action perhaps mimicking or paralleling their <u>in vivo</u> participation in thrombus formation.

For a long time we have been intrigued by the observation that the presence of dextran enhances the polymerization of fibrinogen, at least as measured by the "thrombin time". Even without accounting for dilutional factors in some

in vitro studies, plasma plus dextran plus human thrombin clots faster than does plasma plus human thrombin. If dextran does form a complex fibrinogen, it seems that dextran-fibrin clots should either trap more of the serum proteins or less - but not the same amount as fibrin clots without dextran. Total nitrogen measurements of the clottable protein indicate that the presence of dextran does not influence the size of the clot or its "serum protein trapping" ability. Some refractometry studies have given us some interesting data to compare with those earlier made by Doctor Bloom as regards the serum protein dilutional effect following dextran infusions. These observations may be of great value later in clinical studies to govern the quantity of dextran infused and the interval between infusions. Some of our clinical observations thus far, for instance, indicate a "summation effect" when dextran infusions are spaced by only a few days. The alteration of the dextran molecule to have a carboxy radical on the end rather than an aldehyde has been carried out at Georgia Tech. Enough quantity for in vitro studies has been delivered to us for comparison studies with standard "aldehyde" dextran. Thrombelastographically and otherwise, we are yet to see much difference due to the molecular alteration.

III. PROJECTED STUDIES FOR 1964-1965

With an anticipated completion of the counting facilities and calibration by September, it will be possible to start the in vivo study of formed element and blood vessel surface coating. Comparison of the quantitative difference between damaged and undamaged surfaces will be studied. The time factors of duration of coating in relation to blood concentration will be done.

It is anticipated that the Coulter Counter Studies will be completed in this period in regard to evaluation of this method in the problem of surface coating. The work has already led other workers in the biological field to re-assess the use of the Coulter counter in all size studies. Whatever the outcome of the surface coating studies, the new advances in instrumentation accomplished during last year's grant period have provided a much more accurate and useful method for study of particle or cell size distribution.

The electron microscopy has not as yet revealed the presence of the dextran on the blood vessel surface or, for that matter, on glass. The "end on" studies of blood vessels in the next grant period and the studies which have been initiated of alkaline metal complexing of dextran on the cell surface should define the usefulness of electron microscopy in studying surface coating by dextran.

Carboxy dextran will be studied in vivo and in vitro in regard to blood levels, urine excretion, hemostatic effects. If the characteristics of this molecule are sufficiently similar to dextran, a Carbon-14 tag will be used for further studies. In addition, the carboxyl group will be reduced to an alcoholic group[5] to study the effects on reactivity of the molecule.

Electrical thrombosis is now thought to be the result of electrolysis of water. In the forthcoming period, the gas evolved at the electrodes during

the electrical thrombosis of plasma will be studied using a saline control. The study of the effects of the alterations in pH at the electrode surface have been initiated and preliminary findings show (as might be expected) an increase in H^+ at the positive electrode. The effects of the alterations of oxidation reduction potentials and the sulfhydryl group of the protein molecule are planned.

Studies on hemostasis and coagulation will be continued. The effects of sonic vibration on platelet stability of control and dextran treated platelets will continue. The study of the coagulation mechanism in relation to the in vivo prevention of thrombosis will go on concommitantly with the Carbon-14 studies in dogs.

IV. PERSONNEL

A number of new people are participating in our project:

1. Dr. James P. Isaacs has joined the post graduate and continuing education program of Piedmont Hospital as the Director of Surgical Education and Research. He is working actively in connection with the effect of dextran in the treatment of experimental myocardial infarction. This has required working out a method for producing experimental myocardial infarction in animals[5] so that an adequate model will be available for control and experimental studies.

2. Mr. John Lamb, biochemist and biophysicist, accompanied Dr. Isaacs to Piedmont Hospital from Johns Hopkins University and continues his work on electrical thrombosis in connection with the Hartford Foundation grant.

3. The personnel who have participated in this project at the Georgia Institute of Technology are Mr. R. L. Meek and Mr. Thomas Erb, Electronic Engineers (design of low-level counter electronics), Mrs. Dorothy DeFoor and Mr. Jim Kinard (Coulter counter studies), Mr. John Brown and Mr. Jim Hubbard (electron microscope studies), Mr. Martin Reynolds and Mr. Samuel Fowler have assisted in equipment construction. Dr. Robert Fetner has lent advice and assistance in the Coulter counter studies. Miss Paula Stevenson assisted in the early part of these studies.

V. REFERENCES CITED

1. "Coating of Vascular Surfaces and Cells. A New Concept in the Prevention of Intravascular Thrombosis," Bloom, W. L., Harmer, D. S., Bryant, M. F., and Brewer, S. S., Proc. Soc. Exp. Biol. and Med. 115:384, 1964.

2. "Use of Dextran to Prevent Arterial and Venous Thrombosis," Moncrief, J. A., Darin, J. C., Canizaro, P. C., and Sawyer, R. B., Ann. Surg. 158:553-559, 1963.

3. "Carbon-14 Carboxy-labeled Polysaccharides," Moyer, J. D. and Isbell, H. S, J. of Res. of Natl. Bureau of Standards, 61:71, 1958.

   Isbell, H. S. U.S. Patent 2,653,931, September 29, 1953.

4. "A Simple New Method Producing Selective Coronary Arterial Thrombosis in the Dog," Isaacs, J. P., Bloom, W. L., Lamb, J. C., and Burson, J. L. Submitted for publication to Surgery.

5. "Hydrodextran: Preparation and Study of Blood Level and Excretion Rate," Tyron, Philip F. and Bloom, Walter L., Proc. Soc. Exp. Biol. and Med. 84:462-464, 1953.

VI. PUBLICATIONS

1. "Electrical Thrombosis of Blood Vessels, a Voltage-dependent Phenomenon," Lamb, J. L., Isaacs, J. P., Bloom, W. L., and Harmer, D. S. Submitted to Proc. Soc. Exp. Biol. and Med.
2. "Redesign of the Coulter Counter and Its Application to Biologic Studies," Harmer, D. S., Kinard, J. In preparation.
3. "Mechanism of Dextran Alterations of Hemostasis," Brewer, S. S. and Bloom, W. L. In preparation.
4. "Thromboelastographic Changes Produced by Dextran," Brewer, S. S., Bloom, W. L., Harmer, D. S., Clark, Jeanne. In preparation.
5. "Dextran Treatment of Thrombophlebitis," Bloom, W. L. and Brewer, S. S. In preparation.

A STUDY OF MOLECULAR COATING OF INTRAVASCULAR SURFACES

Interim Progress Report

Project B-254

for the

John A. Hartford Foundation

Covering the period

July 1, 1968 to December 31, 1968

W. L. Bloom and D. S. Harmer

A joint project of the Ferst Research Laboratory of Piedmont Hospital and the Nuclear and Biological Sciences Division

1969

Engineering Experiment Station
GEORGIA INSTITUTE OF TECHNOLOGY
Atlanta, Georgia

A STUDY OF MOLECULAR COATING OF INTRAVASCULAR SURFACES - INTERIM PROGRESS REPORT

For The John A. Hartford Foundation

Covering The Period

July 1, 1968 to December 31, 1968

W. L. Bloom and D. S. Harmer

During the six-month period, July 1, 1968 - December 31, 1968, a number of research problems were investigated. Particular emphasis was placed on investigating the dextran-cell surface interaction and the dextran-plasma (protein) interactions. These studies were directed at determining rates of association-disassociation, equilibrium constants and the effects of temperature both for dextran and carboxy dextran. Since there is some evidence that the binding sites for dextran-carboxy-dextran are similar to those for other weak acids, the interaction of oleic acid with plasma protein and cell surfaces was also studied in an attempt to more clearly define the type and number of binding sites available to dextran and to provide a working model to characterize the system. In addition Scanning Electron Microscope studies of the cell surfaces, with and without exposure to dextran and carboxydextran, were made to see if any significant alteration of the surface of red blood cells occurs under these conditions.

As of July 1, 1968 the Ferst Research Laboratory of Piedmont Hospital was incorporated into the Nuclear and Biological Sciences Division of the Georgia Institute of Technology. This move provides the Ferst Laboratory with more ready access to the Institute facilities and conversely provides the Institute

with access to the Medical community. It is anticipated that this joint enterprise will greatly enhance the interdisciplinary studies in the Biomedical and Bioengineering fields.

The major problem faced during this period has been three complete turnovers in technical assistants for the tracer studies and the required retraining of new personnel. This turnover has slowed down the effective effort with a consequent delay in obtaining results. The Georgia Tech group was fortunate in obtaining the services of two excellent technical assistants for the summer, Mrs. Sue Haycock and Mr. David Schiff. The large amount of experimental data obtained by these two is still being analyzed and will be presented in detail in the final report.

I. TEMPERATURE DEPENDENCE OF CARBOXYDEXTRAN RED CELL EQUILIBRIUM CONSTANT

In order to determine the actual equilibrium constant for the surface adsorption of carboxydextran on red blood cells, the equilibrium experiments need to be run at constant temperature during the entire experiment. Since the basic information desired is the equilibrium constant at body temperature, the experiment should be run at this temperature. However, with the series of manupulations involved, this is difficult with the large number of samples to be processed. By determining the equilibrium constant as a function of temperature, experiments at room temperature can be scaled to body temperature. In addition, from these data, the heat of association can be determined (red blood cell-carboxydextran) and this heat ($\triangle H$) is a direct measure of the bond strength of the carboxydextran cell surface interaction. The $\triangle H$ can then be used to compare with other bond strengths relatively and can also be used to compare with given types of interaction (i.e., hydrogen bonds, weak acid-weak base, etc.) to provide an additional clue to the nature of the surface bonding. Experiments were made at several initial exposure concentrations and at three temperatures. The temperatures were chosen on the basis of practicality for carrying out the entire experiment at constant temperature, and to obtain the widest range possible.

Procedure

The following experimental procedure was run at three different temperatures (5° C, 21° C, 33° C) with the entire experiment (except the actual counting) being conducted in the cold room, the laboratory, and the incubation room, respectively. This was done so the temperature could be maintained constant during the entire separations and incubation procedure.

Eight 1-ml samples of whole blood were obtained from a single donor. These were centrifuged and the red cells were washed three times with normal saline. The samples were then incubated with carbon-14-labeled carboxydextran for 3 hours. (Two samples at each concentration, 2, 3, 4, and 6 grams percent). Each sample of red blood cells was then washed five times with 10-ml normal saline, and one ml of the 5th wash was taken for counting of the supernateust. The remaining red cells were diluted to 10 ml with normal saline and 1-ml aliquoits were removed for counting. Samples were counted overnight. Blood cell counts (conventional RBC/mm^3) were made on the original whole blood and on each of the final diluted samples.

Results

The amount of radioactivity bound to red blood cells of initial incubation solution were linear with concentration at each of the temperatures. This indicates that the association is monomolecular in the carboxydextran (CD) as might be expected in this type of two phase equilibrium (i.e., the CD bound to the cells behaves as if adsorbed on a solid phase, its activity in solution is then unity.) The data for room temperature (21° C) were in good agreement with that obtained earlier at 0.5, 1, 1.5 & 2 grams percent concentration. (Note: in the earlier work 0.25 ml packed washed RBC were taken and 0.5-ml of suspension were counted giving 1/4 the activity/gm percent on the RBC samples) It is also interesting to note that no saturation effects were seen, even at the highest concentrations used (6 gm percent).

From the slope of these activity vs concentration plots the association constants, K_a, were obtained for each temperature. These results are presented in Table I.

It can be readily seen from these results that there is a strong positive temperature dependence in the amount of carboxydextran adsorbed on the red blood cells. The association constants given column one (K_a) are in terms of counts per minute adsorbed per sample per gram percent of carboxydextran. From these data, and the red blood cell count per sample the association constant shown in column 2 (K_a') in terms of molecules of carboxydextran per red blood cell were obtained. The strong temperature dependence indicates a large value for the energy of association. One may use these data in the appropriate integrated form of the Van't Hoff equation*to derive the heat of association $\triangle H$ which is a direct measure of the binding energy.

$$\ln \left[K(T_2)/K(T_1) \right] = \frac{-\triangle H^\circ_a}{R} \left(\frac{1}{T_2} - \frac{1}{T_1} \right)$$

where $K(T_n)$ is the association constant at Tn, with Tn in absolute temperature units, and R is the gas law constant). From the Van't Hoff equation it can be seen that if K_a increases with temperature, then $\triangle H$ is positive and the reaction is endothermic. In the units used here, $\triangle H$ is in kilocalories/mole.

Having found $\triangle H$ from the above equation the association constant may be calculated at other temperatures (i.e., 37° C) as is shown in the last row in columes 1 and 2 of Table I.

* qq.v.: p. 829, "Textbook of Physical Chemistry," Samuel Glasstone, Van Nostrand, New York, (1946).

TABLE I

T(°C)	K_a (1)	K_a' (2)	Cal of △H* from	△H* Method I kilocal/Mole	△H* Method 2 kilocal/Mole
5°	550	4.63×10^3	5° + 21°	+5.716	+5.842
21	978	8.23×10^3	5° + 33°	+5.709	+5.725
33	1420	1.10×10^4	21° + 33°	+5.700	+5.554
37	1598*	1.35×10^4	Average	+5.708	+5.707

* Calculated from the Van't Hoff equation (qq.v.: p.829, "Textbook of Physical Chemistry," Samuel Glasstone, Van Nostrand, New York, (1946).

Method 1. Evaluated using six-place natural log tables.
Method 2. Evaluated using power series expansion of ln x.

(1) In units of dpm per gm percent carboxydextran

(2) In units of molecules of carboxydextran per red blood cell per gm percent carboxydextran.

TABLE I

T(°C)	K_a (1)	K_a' (2)	Cal of △H* from	△H* Method I kilocal/Mole	△H* Method 2 kilocal/Mole
5°	550	4.63×10^3	5° + 21°	+5.716	+5.842
21	978	8.23×10^3	5° + 33°	+5.709	+5.725
33	1420	1.10×10^4	21° + 33°	+5.700	+5.554
37	1598*	1.35×10^4	Average	+5.708	+5.707

* Calculated from the Van't Hoff equation (qq.v.: p.829, "Textbook of Physical Chemistry," Samuel Glasstone, Van Nostrand, New York, (1946).

Method 1. Evaluated using six-place natural log tables.
Method 2. Evaluated using power series expansion of ln x.

(1) In units of dpm per gm percent carboxydextran

(2) In units of molecules of carboxydextran per red blood cell per gm percent carboxydextran.

The values of ΔH obtained are given in Table I. The two methods used reflect the sensitivity of the function to the accuracy of the calculation and is worthy of note because of the agreement of the averages. This also indicates that the relative K_a's at different temperatures are in good agreement with each other, otherwise widely different values of ΔH would have been obtained.

The fact that the association constant increases with temperature (or equivalently ΔH is positive) is a significant datum in that it is clear evidence that the bonding is <u>not</u> hydrogen bonding. The number of molecules bound by hydrogen bonding decrease with increasing temperature. (K_a decreases with T, and ΔH is negative). The magnitude of ΔH tends to indicate that a single bond, rather than many, per carboxydextran molecule is involved in the bonding to red blood cells. This is consistent with the earlier observations that carboxydextran bonds more strongly than does ordinary (aldehyde) dextran and that the carboxyl or the (aldehyde)group respectively are responsible for the bonding rather than the many alcoholic groups of the molecule. From the observations that a stronger acid (oleic acid) can displace either dextran from cell surface, and these results it strongly suggests the thesis that the bonding is an acid-protein-amine base reaction. (Note that aldehydes act as very weak acids).

In view of the ability of both dextran and carboxydextran to prevent thrombosis at damage sites in blood vessels, and the longer persistance of carboxydextran on surfaces, and probably on blood proteins in general, at lower blood levels than dextran, it is strongly indicated that carboxydextran should be used in treatment of thrombophlebitus and other chronic clotting problems. It is suggested that the anti-thrombiotic effect would persist longer than with dextran and certainly longer than with heparin and without the dangers of heparin. Studies should be made on the long term anti-thrombiotic effect of both dextran and carboxydextran.

II. AMOUNT OF CARBOXYDEXTRAN ATTACHED TO RED BLOOD CELLS

From the data obtained in the constant temperature experiments, some interesting observations can be made, particularly about the amounts of carboxydextran in solution and on the cell. Using the data from a sample incubated with 6% carboxydextran at 21° C as an example:

After 5th wash: red cell count in 10 ml suspension
= 3.82×10^5 RBC/mm^3

Radioactivity in 1 ml of suspension
= 5320 dpm

Radioactivity in 1 ml of 5th Wash
= 920 dpm

Hematocrit = 6

From the hematocrit and the activity in the wash solution, it is readily determined that less than 20 dpm of the activity in the RBC results from trapped wash in the centrifuged RBC after the 5th wash. Neglecting this small amount, the activity adhering to the Red Blood Cells is (per cell)

$$(5.32 \times 10^3 \text{dpm/ml})(1\text{-ml}/3.82 \times 10^8 \text{ RBC}) = 1.39 \times 10^{-5} \text{ dpm/RBC}$$

or

$$0.627 \times 10^{-14} \text{ millicurie/RBC}$$

Since the specific activity of the carboxydextran is 1-mc/gm, this corresponds to 0.627×10^{-14} gm of dextran/RBC. The average molecular weight is 75,000, therefore this weight corresponds to 5.03×10^4 molecules of carboxydextran per red blood cell. Comparison to the original amount of carboxydextran shows that 0.004% has adhered to the red blood cells after five 10-ml saline washes.

III. PROCEDURE STUDIES: RED BLOOD CELL-DEXTRAN EQUILIBRIUM EXPERIMENTS

In the sample preparation of mixtures containing red blood cells and/or plasma protein it is necessary to solubilize the cells and/or protein before counting in the liquid scintillator mixture: the color from the hemoglobin must be removed or the light from the scintillations will be highly absorbed resulting in too much quench. Frequently, it was observed that an abnormally large variation occurred in the observed tracer counts with successive duplicate runs. These variations were believed to be in excess of the combined experimental errors due to pipeting, volume of cell measurements, counting statistics, etc. Several studies were made to determine if this was due to the incomplete solution of the cell protein. It was found that twice as much solubilizer was needed as had been recommended by the manufacturer (Nuclear-Chicago). This decreased the count-efficiency variation with time but did not eliminate it. The following series of procedures was then tried to determine a standard procedure.

In each case one-ml samples of whole blood were taken, the red blood cells washed three times with normal saline, the cells separated each time by centrifugation. The cells were then incubated with carbon-14 labeled carboxy-dextran for three hours and washed five times with normal saline. The cells were resuspended in 10 ml of normal saline and one-ml aliquots of the suspension were processed by the following methods:

Procedure I

To one-ml of the red blood cell suspension in a standard sampling vial was added 3-drops of 50% H_2O_2 at 5 minute intervals. The suspension was allowed to stand 20 minutes to decolorize. Two-ml of NCS were then added and the solution

allowed to stand for 90 minutes to solubilize. Fifteen-ml of tolulene based scintillator was then added and the solution counted after 2-minute dark adaption.

Procedure II

To one-ml of the red blood cell suspension was added 2-ml of NCS and the solution was allowed to stand 15 minutes. Five drops of 50% H_2O_2 were added and allowed to stand for 90 minutes after which 15-ml of the toluene scintillator were added and the solution counted.

Procedure III

Two-ml NCS was placed in the counting vial and 5 drops of 50% H_2O_2 were added. One ml of the red cell suspension was then added and allowed to stand 90 minutes. The sample was then counted.

The most often encountered problem in liquid scintillation counting, chemi- or photo- luminescence did not seem to be the cause of the variations on investigation. Dark adaption for 2 minutes before counting was sufficient to remove the photo-luminescence, and chemical reactions producing light in the scintillation mixture were not significant. However, it was observed that the count rate did vary as a function of time after preparation as evidenced by the quench ratio and the observed net counting rate. One of the primary causes of this variation was the amount of solubilizer (NCS) added. It was observed that, when counting red blood cells, variations in the corrected disintegrations-per-minute (dpm) of 30% could occur over a period of time (13 hours). This was not observed for samples not containing red blood cells (washes, etc.).

Each sample was counted several times in succession; immediately after preparation, and at intervals up to 13 hours.

It was found that Procedure I gave satisfactory, unchanging efficiency corrections after two hours whereas the others were still changing after thirteen hours. The waiting periods between adding NCS and the peroxide (H_2O_2) and the toluene are critical to the more rapid stabilization of the counting efficiency. In fact, it is recommended that the samples be prepared by Procedure I and allowed to stand overnight (approximately 12 hours) before counting to ensure stabilization. It appears that the culprit in the changing efficiency is the oxygen evolved from the reaction of the hydrogen peroxide with the biological material. The waiting period after addition of the H_2O_2 to allow the excess oxygen to evolve seems to solve the problem completely. Addition of the peroxide after the NCS requires more peroxide to decolorize due to the raction of some of the peroxide with the NCS.

IV. FAT BINDING TO PROTEIN AND PROTEIN SURFACES

Studies were continued in this period on the interaction of fatty acids with proteins. Most of the effort was devoted to investigating the equilibrium of fatty acids with plasma protein. A clinical procedure is being developed for determining the relative additional fatty acid that can be bound by plasma protein. The "nearness-to-saturation" appears to differ from individual to individual, and may be indicative of the tendency of fatty deposits in blood vessels and other diseases. The procedures reported earlier were investigated further, particularly the use of ion-exchange resins to remove unbound fatty acid from plasma-fatty acid mixtures. The intent of this procedure is to separate unbound fatty acid from solutions containing plasma and fatty acid in order to determine the amount of fatty acid bound to plasma protein. After exposure of plasma to fatty acid, an ion exchange resin is added, the mixture agitated and incubated for various times, and the resin is allowed to settle. The solution then contains essentially only protein bound fatty acid. By using radioactively labeled fatty acid, the amounts bound to resin or to protein can be determined.

The primary problem investigated was whether the addition of the resin significantly changed the amount of fatty acid bound to the protein:

In the original reaction,

Fatty acid + protein $\rightleftarrows$ protein-bound fatty acid

$$FA + P \rightleftarrows FAP$$

The resin-fatty acid interaction

$$R + FA \rightleftarrows FA\text{-}R$$

The overall reaction with both present:

$$\begin{array}{l} FA + P \rightleftarrows FA\text{-}P \\ + \\ R \\ \Downarrow\Uparrow \\ FA\text{-}R \end{array}$$

If the bonding to protein is much stronger than to resin, and if the rate of dissociation of protein-bound fatty acid is slow, and the rate of association with the resin is fast, then adding the resin will not cause a significant shift in the protein-fatty acid equilibrium.

The resins studied were Amberlite, Dowex, and Rexyn. The reaction rates with aleic acid were slowest with Amberlite, next slowest with Dowex, and fastest with Rexyn. The amounts bound per unit weight were largest with Dowex, and least with Amberlite.

These results are applicable to the procedure for determining the number of acid binding sites both on plasma protein and on red blood cells. It is of interest to determine the number of binding sites and their relative strength of bonding in this manner to compare with the number of sites available for dextran bonding. These studies are continuing and the details will be given in the final report. A large body of data has been collected and is being evaluated currently.

V. SCANNING ELECTRON MICROSCOPE STUDIES

With the advent of the Scanning Electron Microscope which allows visualization of surfaces and surface contours at high magnification, it was of interest to see if the cell surface interaction phenomena could be seen in any manner. The electron microscope group at Georgia Tech, directed by Mr. John L. Brown, Senior Research Physicist, has recently obtained a Phillips Scanning Electron Microscope, Model EM-200, with a maximum resolution of 7 Angstroms and maximum practical magnification of 2×10^6 X. This is an addition to the several conventional transmission-type electron microscopes which had been used earlier in the dextran studies (see Annual Progress Report 1966). The scanning electron microscope has the ability to give a representation, on film, of objects over a wide range of magnification that is essentially the same as would be seen by conventional photography of macroscopic objects; that is, although two-dimensional in nature, the third dimension is indicated by shadow-like effects, in contrast to the conventional transmission only pictures. Since the surface and surface contours are visualized in this manner, it was felt that perhaps the effects of the surface absorption of macro-molecules, particularly dextran and carboxy-dextran, could be observed on the red blood cell itself, which could be used as a prototype for the cell-surface coating studies. It was also possible that an extremely long molecule, such as dextran, if arranged in a regular manner on the cell surface, could be seen either as producing an apparent cell size increase, or by masking the finer detail of the surface. It was also suggested that some distortion or change of the cell shape might result from this macro-molecular coating.

As is always the case with electron-microscope sample preparation, great care must be taken to avoid introduction of artifact by sample handling and

and preparation. This is particularly true when one is dealing with cells, especially those as fragile and distortable as red blood cells. In addition, it should also be noted that a "training phase" is necessary in such a study in that one must learn the correlation of what one "sees" with an electron micrograph with what one has learned to see in conventional optical microscopy. In an attempt to avoid some of these pitfalls, Miss Jean Clark, hemotology technologist, prepared all the samples for electron microscopy and at the same time observed parallel samples with the more familiar optical techniques. The transmission-type electron microscope was also used with similar samples. This technique was very fruitful in detecting the introduction of artifact during sample preparation. These results are described below. Several sample preparation techniques were tried and from our experience it is obvious that considerably more extensive research is needed in this area when dealing with cells such as red blood cells.

Procedures:

Sample Preparation - Red Blood Cells for Scanning Electron Microscopy

In view of the large number of variations in sample preparations tried, a summary listing of the samples is given below. In each case fresh, whole human blood samples were taken, and if applicable, centrifuged and washed three times with normal saline. Some samples were made with whole blood. Samples were incubated with dextran in saline, in sucrose, saline alone, both with and without anti-coagulation agents such as EDTA or citrate, etc. Various freeze-drying techniques were tried and various shadowing techniques and materials were tested. Samples were also prepared by simple air evaporation of the water.

	Anticoagulant	Specimen	Exposed to: Normal Saline	Dextran in Saline MW = 75,000	Dextran in Saline MW = 26,000	Dextran in Deptrose MW = 75,000	Non Incubated	Incubated
1	EDTA	washed rbc	X	X	X	-	X	X
2	NONE	washed rbc	X	X	-	X	X	X
3	CITRATE	whole blood	X	X	-	X	X	
		washed rbc → from above 3	3	3	-	3		
4	CITRATE	whole blood	X				X	X
5	NONE	platelet rich plasma		X				X
6	NONE	No cells	X	X		X		
7	CITRATE	Plasma						
8	CITRATE	-	-	-	-	-	-	-

Samples Studied With
Scanning Electron Microscope

Anticoagulant	Specimen	Exposed to: Normal Saline	Exposed to: Dextran in Saline MW = 75,000	Exposed to: Dextran in Deptrose MW = 75,000	Non Incubated	Incubated
[1]CITRATE	whole blood				X	
	washed rbc from 1				X	
[2]CITRATE	whole blood	X	X	X	X	
	washed cells→ from above 2	2	2	2		
[3]CITRATE	platelet rich plasma		X		X	X
[4]	no cells	X	X	X		

Samples Studied with
Transmission Electron Microscope

Conclusions:

In spite of the large number of techniques tried, no clear correlation of surface appearance with the presence or absence of dextran could be obtained. In fact, two effects were seen to predominate in the appearance of the samples, both having to do with sample preparation. The manner of washing of the red blood cells and the incubation time have a major effect on the cell appearance. Also the method used for removing the water surrounding the cells prior to inserting the samples into the high vacuum necessary for electron microscopy, has a major effect on the cell appearance. Since the cells, while in solution, must have nearly the correct, normal saline concentration to avoid hemolysis, then during evaporation of the water the saline concentration must increase to saturation, causing some cell damage (frequently rupture). In any case, the hydroscopicity of sodium cloride tends to remove water from the cell itself, causing apparent changes. This is also the disadvantage of freeze drying. If after any of the water removal techniques, the cells are exposed to humidity, then the small amount of water adsorbed will create a concentrated saline solution at or near the cell surface causing some distortion. These two problems are difficult to overcome and more study is needed in this area. On reviewing the entire series of photographs taken, the overall impression is that exposure to dextran tends to preserve the appearance of the red blood cell. It is of interest to note however that, in spite of all the difficulties, several techniques were developed which look promising for the visualization of red blood cells and even platelets at very high magnification. Sample photographs are shown in the Appendix of red blood cells at various magnifications. A considerable amount of detail can be seen in these photographs. However, some more extensive study is needed to determine which are due to artifact and which are

due related to the real surface appearance. As is always the case, what one would really like to know is the appearance of the cell in vivo rather that in vitro, particularly when the cell has been multiply processed and studied in the absence of any surrounding fluid. In conclusion, although unable to clearly determine the surface effects of dextran or being able to visualize dextran on the surface of the cell, the scanning electron microscope shows great promise for this purpose. Considerable study is needed in sample preparation.

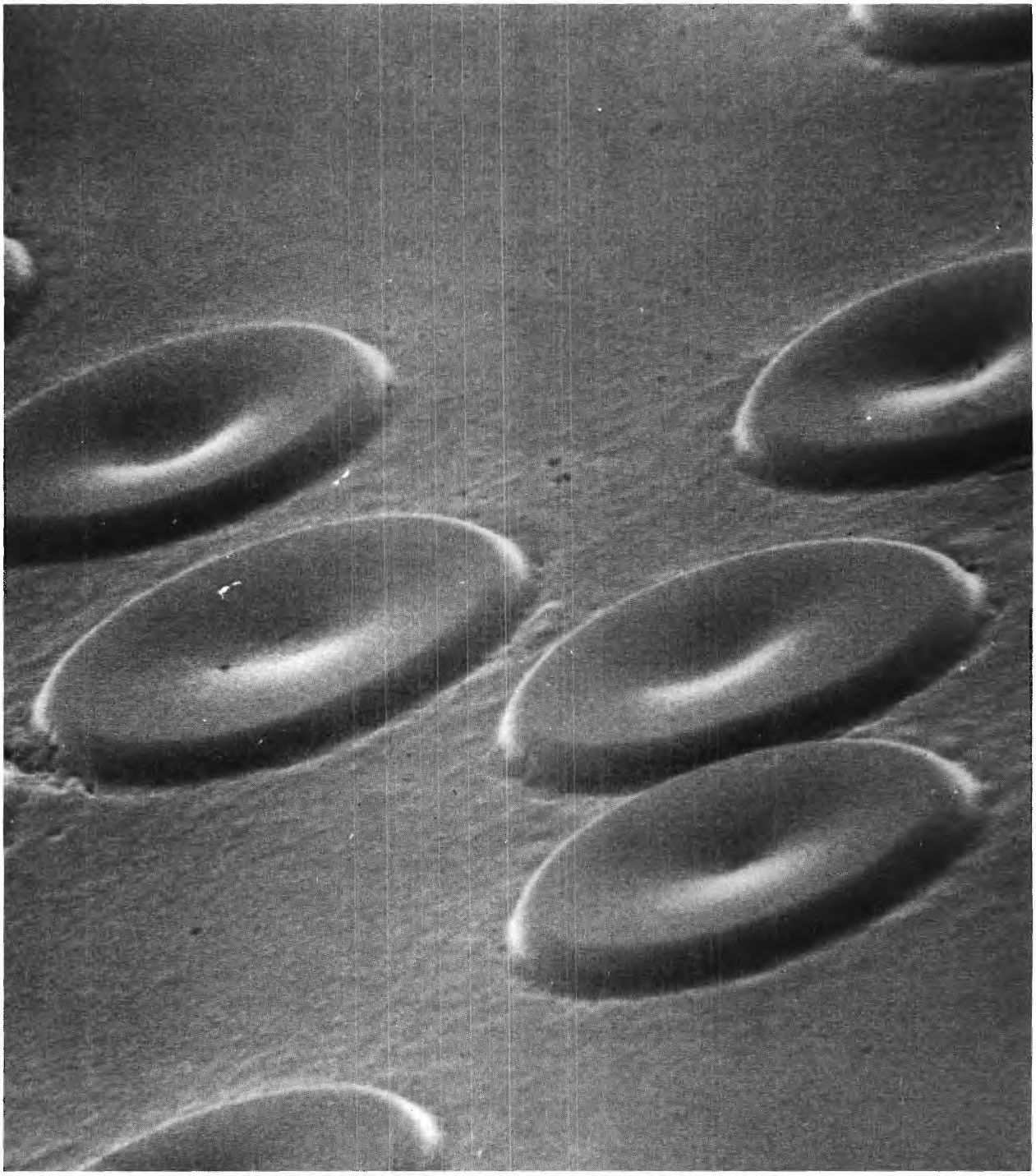


FIGURE 1. WHOLE BLOOD with CITRATE ANTICOAGULANT - INCUBATED at 37° for 30 minutes - MAGNIFICATION 9360X

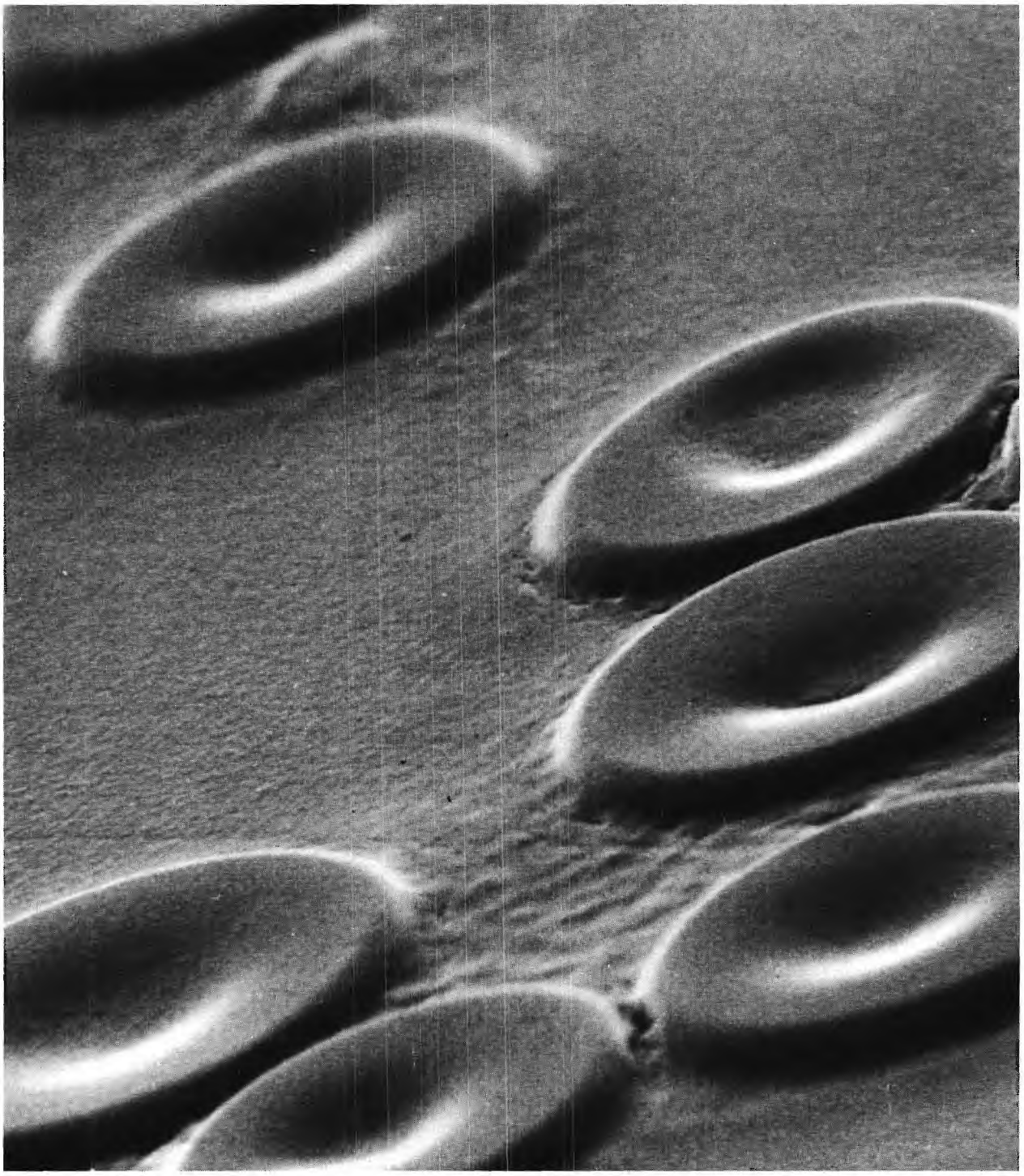


FIGURE 2. WHOLE CITRATED BLOOD - 0.9 ml INCUBATED with 0.1 ml 6% DEXTRAN (MW 75,000) in SALINE for 30 minutes at 37°C - MAGNIFICATION 10,274X

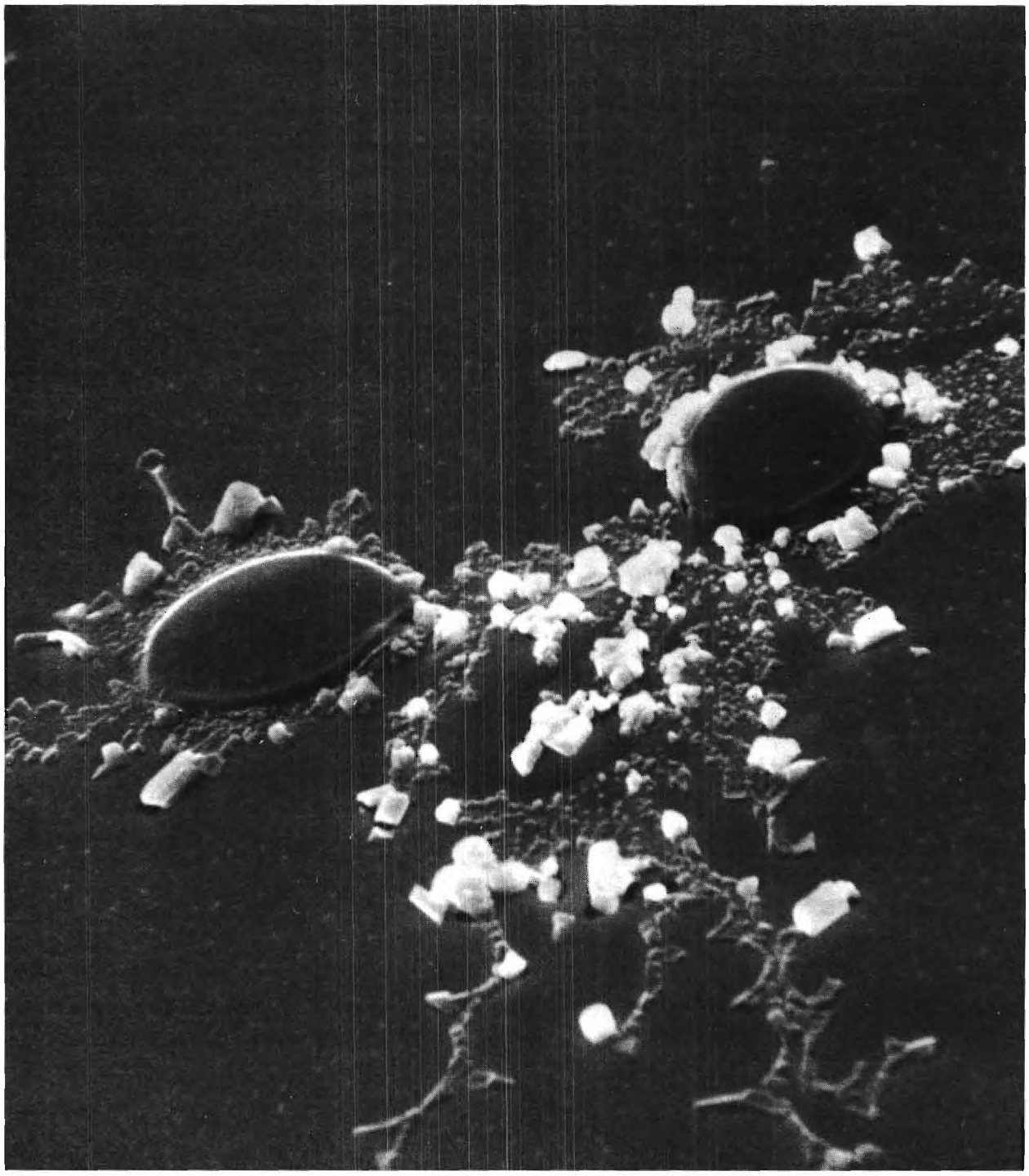


FIGURE 3. SALINE WASHED RED BLOOD CELLS - INCUBATED 2.5 hrs at 37°C with 1 ml 6% DEXTRAN (MW 75,000) in SALINE

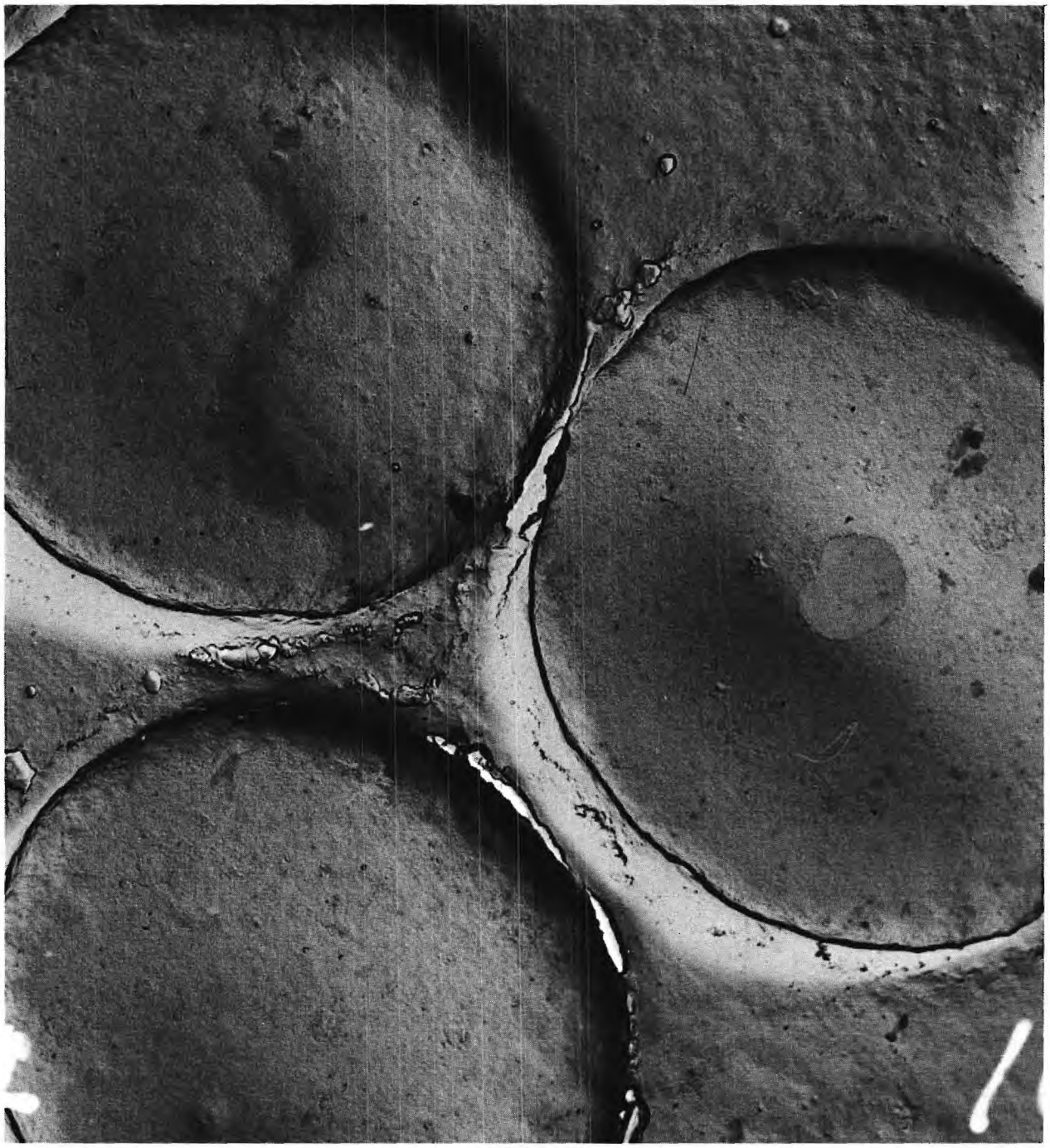


FIGURE 4. WHOLE CITRATED BLOOD - 0.8 ml with 0.2 ml NORMAL SALINE - TRANSMISSION ELECTRON MICROGRAPH - MAGNIFICATION 12,800X

FIGURE 5. WHOLE CITRATED BLOOD - RED BLOOD CELL SURFACE in FIG. 4
MAGNIFICATION 192,000X

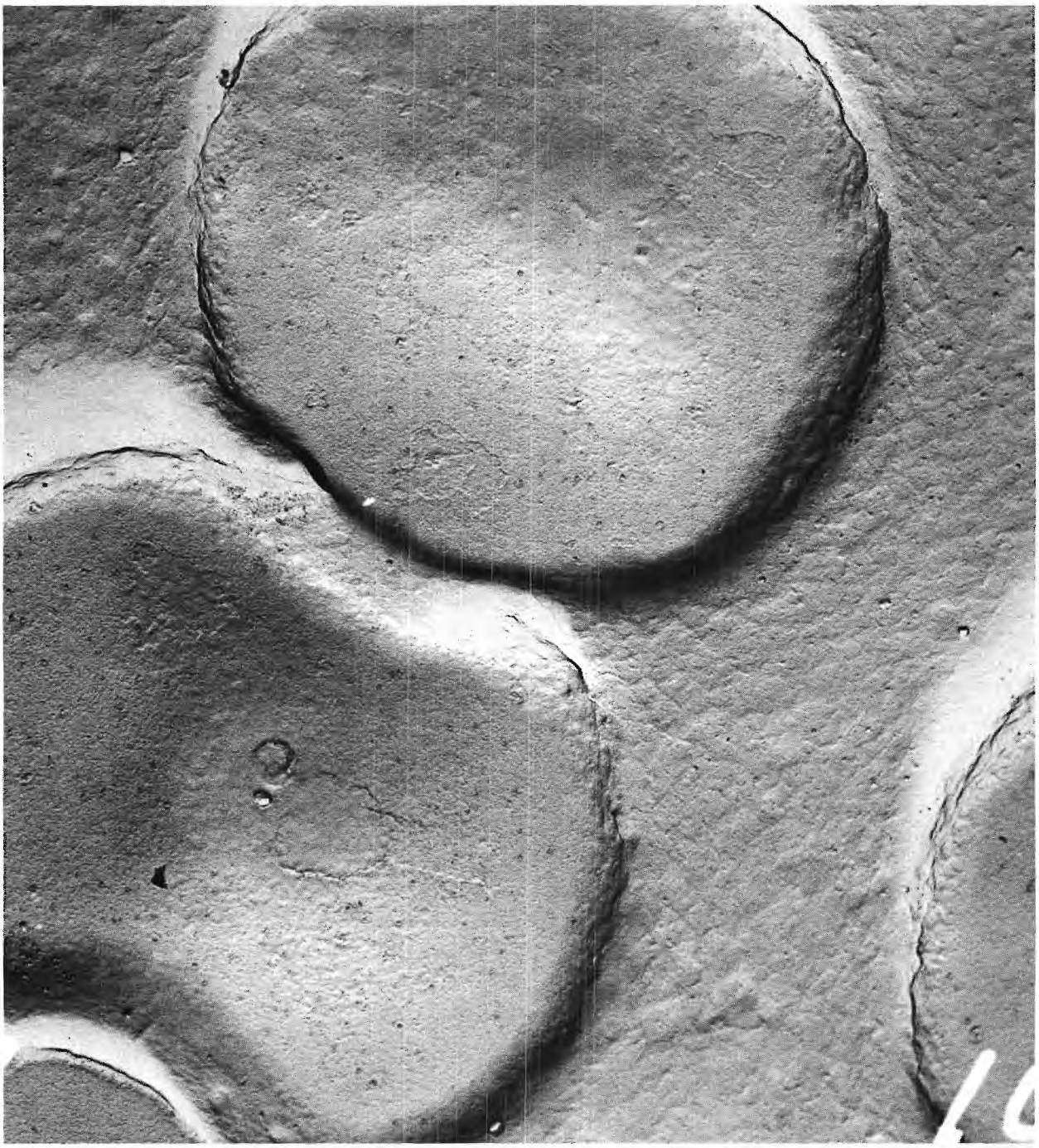


FIGURE 6. WHOLE CITRATED BLOOD - 0.8 ml with 0.2 ml 6% DEXTRAN in SALINE. TRANSMISSION ELECTRON MICROGRAPH MAGNIFICATION 12,800X

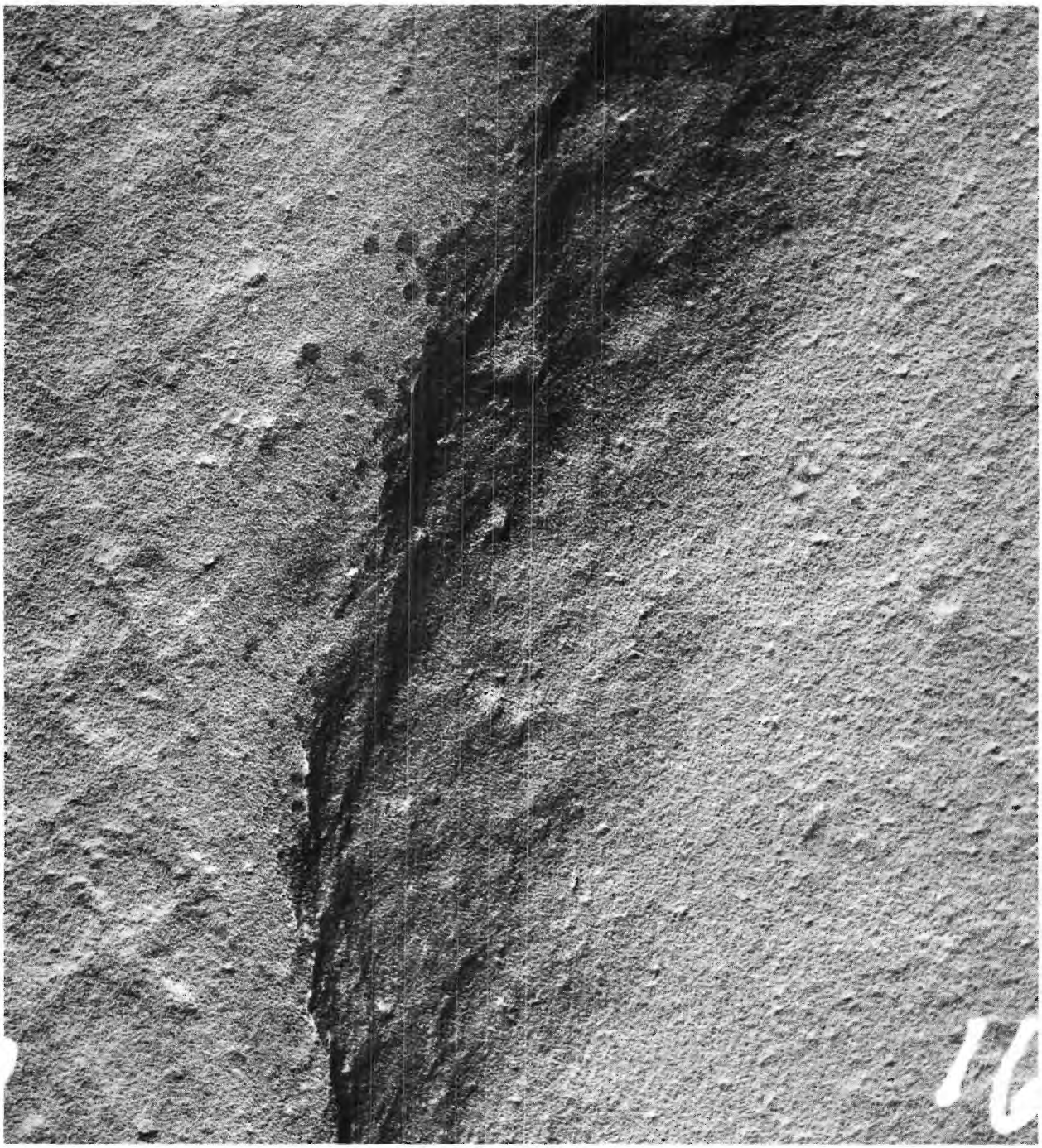


FIGURE 7. WHOLE CITRATED BLOOD - CLOSE-UP of CELL SURFACE in FIG. 6.

FIGURE 8. WHOLE CITRATED BLOOD RED BLOOD CELL SURFACE - 0.8 ml with 0.2 ml 6% DEXTRAN in DEXTROSE - TRANSMISSION ELECTRON MICROGRAPH - MAGNIFICATION 192,000X